A SILVER IMPREGNATION METHOD FOR PERIPHERAL NERVE ENDINGS*

RICHARD K. WINKELMANN, M.D.

The silver ion is a most important histologic tool but one that is little understood. In view of the meager knowledge of the physicochemical forces involved, it is difficult to recognize the necessity for adding to the long list of empiric methods for silver impregnation. However, the method to be described has advantages provided by no other histologic method. It is proper that these advantages should be outlined as an introduction to a description of the procedure and as a justification for its publication.

ADVANTAGES OF PROPOSED METHOD

Definition of Nerve Structure.—The contrast between nerve fibers and connective tissue leaves no doubt as to the neural identity of the structures in question. This method impregnates both myelinated and nonmyelinated nerve fibers (fig. 1). The fine detail of various forms of nerves can be clearly shown, as demonstrated by the photomicrograph of the basketlike endings in a Meissner's corpuscle (fig. 2). The method stains nerve structure selectively so that the nerve patterns may be followed over great distances and through many planes of focus in sections cut as thick as 200 microns. This method employs frozen sections and distortion is minimized. If the sections are mounted permanently, after fixation in formalin, shrinkage of tissue occurs.

The method is simple. All technicians should be able to produce good sections with a little practice. The time involved in the method is 6 to 7 hours, which allows sections to be completed in a single working day. The method works well with several routine fixatives.

Reproducibility.—One of the serious defects of methods for impregnation of silver has been the capricious nature of the technics. They give both good and bad results under apparently constant conditions. In addition, few people have been able to perform these technics in a manner so as to produce sections which allow interpretation. This method achieves a degree of constancy by controlling the temperature of impregnation and of reduction. It has been possible to produce excellent sections for more than 2 years and in three laboratories.

* Now on duty with the Venereal Disease Division, U. S. Public Health Service. At present working in the laboratory of Dr. James O. Foley, Department of Anatomy, Medical College of Alabama, Birmingham, Alabama. The author wishes to acknowledge the help in the form of encouragement and facilities furnished by the following people: Drs. A. I. Lansing and E. W. Dempsey, Department of Anatomy, Washington University, St. Louis, Mo.; Drs. P. A. O'Leary, Hamilton Montgomery and J. W. Kernohan, Sections of Dermatology and Syphilology and of Pathologic Anatomy of the Mayo Clinic, Rochester, Minn.; Drs. James O. Foley and T. E. Hunt, Department of Anatomy, Medical College of Alabama, Birmingham, Ala.

The Mayo Foundation is a part of the Graduate School of the University of Minnesota.

Received for publication August 20, 1954.

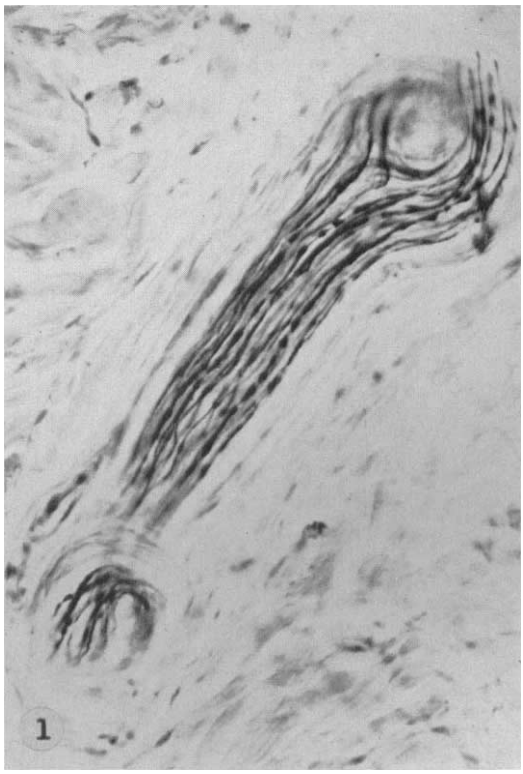

FIG. 1. A section 75 microns in thickness showing a nerve trunk in the dermis of a human palm as seen from above. It contains both myelinated and nonmyelinated fibers that separate and cross at one end and that make a 360 degree turn at the other end (×180).

Photography.—The literature contains no clear illustration of the cutaneous sense organs in man. The method to be described allows unequivocal presentation by photomicrographs of the bodies that are present. It should be possible to catalogue the sensory end-organs as to type and location for comparison of exact structure with physiologic function. Because of the great contrast in the sections, it is possible to photograph several planes of focus individually and print them simultaneously to achieve a full pattern of the nerve or ending. This process was followed in the production of figure 3.

METHOD

Materials.—These include small glass dishes, glass rods, a drying oven that may be set at 55°C, a constant temperature bath, a 100 ml mixing, graduated cylinder and equipment for cutting frozen sections. All glassware must be cleaned scrupulously. Immersion in bichromate cleaning solution for 2 hours or more, followed by thorough rinsing with distilled water, is a good routine.

Reagents—All the following reagents must be Analytical Reagent grade

FIG. 2. A section 25 microns in thickness of a human finger pad showing fine detail of a Meissner's corpuscle. This has a great resemblance to the terminals of some hederiform endings of Ranvier (×1,000).

materials, unless otherwise specified. No chemicals should be used if any signs of discoloration or deterioration are present.

1. Urea; the type in use is made by the Baker and Adamson Company.
2. Silver nitrate.
3. Pyridine.
4. 95% Ethyl alcohol; made from U.S.P. absolute alcohol.
5. Mercuric cyanide.
6. Picric acid.
7. Hydroquinone, purified crystalline.
8. Sodium sulfite, anhydrous. Differences are apparent among various brands; the Mallinckrodt reagent is used at present.
9. Gold chloride.
10. Sodium hyposulfite.

Fixation.—The method works well with both neutral and simple 10% formalin fixation. It also works well with materials fixed in Bouin's solution. The material to be used is kept in the fixative until sections are cut. If Bouin's fluid is used, the material should be used immediately after adequate fixation. Helly's fluid allows only faint impregnation.

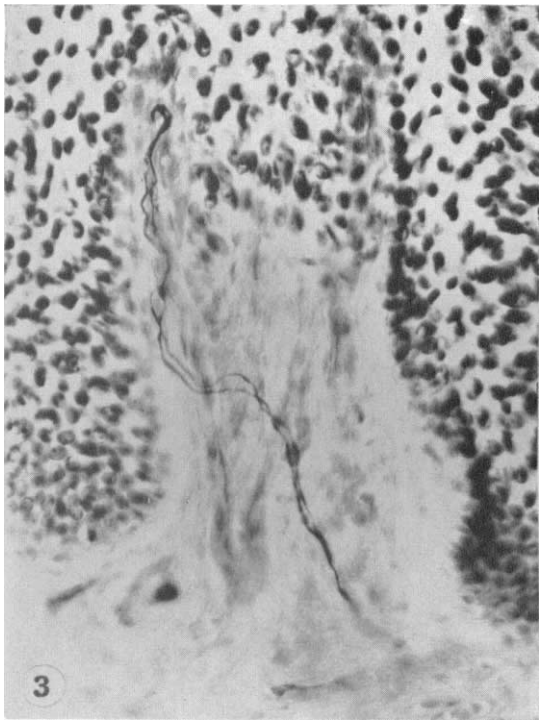

FIG. 3. A section 25 microns in thickness of a human finger pad showing a papillary nerve. The nerve lies in two planes of focus, the lower half being in a more superficial plane. In order to provide an illustration of the continuity present in the section, the two parts were photographed on separate negatives. The negatives were then superimposed and printed to give the resulting photomicrograph (×300).

Technic.—1. Frozen sections are cut at the desired thickness and placed in distilled water until the impregnating solution is made. At all times clean glass rods with bent tips are used to accomplish transfer of the sections.

2. The impregnating solution is made as follows:

a. Weigh out 10 gm. of urea. This amount is not critical; ±1 gm will not affect the results. Place the urea in a graduated cylinder and add 1% solution of silver nitrate in distilled water up to the 30 ml mark. Dissolve by agitation. If the solution becomes cloudy, the particular sample of urea is of no value for the method.
b. Add 18 ml of 95% ethyl alcohol.
c. Add 1.6 ml of pyridine.
d. Add 0.1 ml of a solution containing 1% mercuric cyanide and 1% picric acid in distilled water.

3. Place the impregnating solution in a glass dish with a ground-glass cover and add the sections to it. Place the dish in the oven at 55°C for 5½ hours.

4. At the end of this time, remove the sections from the impregnating bath and carry them through the following procedures:

a. Agitate in 95% ethyl alcohol for 5 seconds.

b. Reduce in a solution of 1% hydroquinone and 5% anhydrous sodium sulfite in distilled water to which 30% by weight of urea has been added. The addition of the urea should be done at the time the reduction is to be accomplished. A simple way to do this is to put 10 gm of urea in a graduated cylinder and add the stock reducing solution up to the 30 ml mark. The reducing solution must be made at least every third day. The reduction will take place in about 1 minute. The temperature must be kept constant at whatever value will accomplish this (see comments on technic).
c. Transfer through two changes of distilled water; 1 minute in each of two dishes is an adequate period.
d. Tone for 10 minutes in 0.2% gold chloride in distilled water containing 25 drops of glacial acetic acid per 100 ml.
e. Transfer through two changes of distilled water as just described.
f. Place in 5% sodium hyposulfite in distilled water for 10 minutes.
g. Place in distilled water until it is convenient to mount them.
h. Mount in glycerin or, for permanence, in a resin medium, as clarite or permount, after dehydration and clearing.

COMMENTS ON TECHNIC

This is a discussion of the manner in which the variables of the procedure may be controlled. The method may be varied with respect to time, temperature, ionic concentration, pH and technic to give different or the same results.

Impregnation.—The composition of the impregnating fluid has not been varied successfully, although it must be possible to simplify the mixture. It is not necessary to use 55°C. as the impregnating temperature. Temperatures as high as 65°C. have been used with success, but it is considered that the best range is 50 to 55°C. In general, the higher the temperature the more quickly the impregnation is accomplished. This observation can be used to plan the most convenient time for the laboratory work. The temperature of 55°C. is chosen to give good impregnation in time to finish the sections on the same day they are cut.

Brands of urea vary and bottles of the same brand show a variation. Urea that produces good sections at one time may not do so a month later. What this variable is has not been determined. Addition of urea to the silver nitrate may produce a precipitate much like silver chloride or cyanide; it may be that this is the result of simple contamination with these ions in spite of manufacturers' statements and care in the laboratory. It is known, through studies done by Dr. C. H. Winkler, Jr., of the Department of Bacteriology of the Medical College of Alabama, that contamination with urea-splitting or other bacteria is not a factor.

Reduction.—It has been found that the reduction produces the best results when it proceeds at a definite, controlled rate. The higher the temperature, the more rapid the rate of reduction. After addition of urea, the reducing solution rapidly turns cold. If it is brought to the optimal temperature and maintained there, the reduction proceeds at a constant rate. The best sections are produced when the reduction is accomplished in about 1 minute. The optimal temperature for this rate of reduction has a narrow zone, usually of a range of 1°C. Unfortunately this optimal temperature varies with the sample of urea in use. For example, the urea in one bottle produced sections that were reduced best at 26° to 27°C., while the next batch produced optimal reduction at 31° to 32°C.

If the temperature of reduction is too high, the reduction speeds past the point of good histologic contrast and excessive cellular staining takes place before the process can be stopped. If the temperature is too low, the reduction does not take place in periods up to 15 minutes, and extraneous silver precipitates will form. By control of the temperature, it is possible to stop the reduction when the structures of interest have been stained selectively. In skin the following order is maintained: myelinated nerve; nucleoli of epidermal cells; nonmyelinated nerve or capillaries or both; muscles; sweat and sebaceous glands; connective-tissue cells and connective-tissue fibers. In most instances, the largest myelinated nerves and the dendritic cells are visible without any reduction process.

In working with the skin, one may find the color of the epidermis helpful as a guide to control of the depth of reduction. As reduction proceeds, the epidermis turns dark brown or black over the range in which the nerves are being stained selectively. It is easy to stop reduction too soon and so miss the finest fibrils. Thus, with every group of sections, it is wise to develop several control sections and mount them in glycerin for inspection. These pilot sections can be used until, through practice, one is able to recognize good reduction grossly as it occurs. When changing from one tissue to another or from one fixative to another, it is usually necessary to change the temperature and time of reduction in order to get the best possible contrast and staining.

The role of the temperature of reduction in control of the results of silver impregnation is thus important. In preliminary work, it appears that an optimal temperature also obtains for the Bodian method for a given set of impregnating conditions. The general applicability of this fact is being tested in other methods with Dr. J. O. Foley.

COMMENT

This method follows the basic scheme of the Cajal frozen-section method from which it developed (1). It utilizes the principle of increasing contrast in silver methods by means of urea, which was first used in a technic developed by Ungewitter (2). It differs from both methods in the results produced and in the manner in which the conditions of the process are controlled.

The mechanism of action of urea in this instance is not known. Two ideas appear reasonable. It is possible that the urea increases the penetration of tissue by ions, in this case the silver ion, as suggested by McClung, quoted by Lee (3). A better explanation may be that the urea provides an increase in the specificity of the impregnation and a retardation of the rate of reduction by the formation of urea-addition compounds with the connective tissue. This type of action has been shown for urea with respect to aliphatic compounds and fatty acids (4).

The impregnating fluid is always alkaline. It has been possible to confirm the work of Holmes (5), and it was on this basis that the pH was kept well above 7. In comparison with many methods for sensory nerve endings, the concentration of silver ions used is small. This too is based on the studies of Holmes. It is probable that the number of silver ions necessary may be far less than the

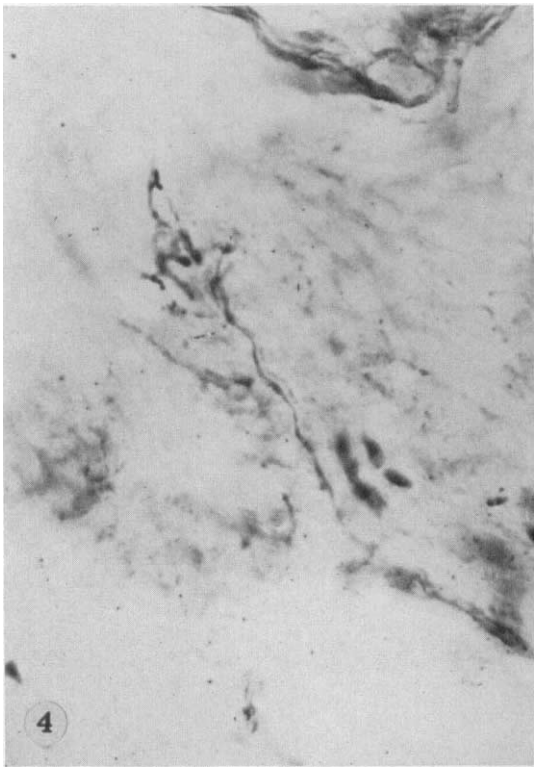

FIG. 4. A section 50 microns in thickness of a human prepuce from a newborn showing a papillary nerve arising in a faintly outlined papilla. The nerve ends adjacent to a group of dendritic cell processes. The subepidermal connections of this nerve are slightly out of focus in the lower right (×360). All sections were made from tissue fixed in simple 10% formalin.

number in use. No compromise in concentration of silver ions or in time and temperature of impregnation and reduction has proved as successful as the method outlined in this communication.

The method was developed during a search for a way to demonstrate both nerves and dendritic cells. The results of this may be seen in figure 4. It has not been possible to make statements about the relationship of these structures prior to this time. A study of this problem is in progress.

The most remarkable accomplishment of this method is that it allows the observation of thick sections. To my knowledge, there is no other method of silver impregnation that shows nerve fibers clearly over wide areas and through depth. Studies in progress (and in press) have indicated that use of this method will expand the field of investigation to which methods employing methylene blue have been applied (6). Because of its ease, adaptability and reproducibility, it may be hoped that this new method will prove of value to all interested in cutaneous innervation.

SUMMARY

A method for silver impregnation has been presented that has the advantages of clarity, ease of technic, a degree of reproducibility and the ability to utilize thick sections of tissue in which neural structures may be followed through depth and over distance and photographed with distinctness. It is hoped this method will aid in clarifying the morphology of sensory endings.

REFERENCES

1. Ramon-Cajal, S.: Histology. Baltimore, William Wood & Company, 1933.
2. Ungewitter, L. H.: Urea silver nitrate method for nerve fibers and nerve endings. Stain Technol., **26:** 73–76, 1951.
3. Lee A. B.: The Microtomist's Vade-Mecum; a Handbook of the Methods of Microscopic Anatomy, Ed. 8. Philadelphia, P. Blakiston's Son & Co., 1924.
4. Schlenk, Hermann and Holman, R. T.: The urea complexes of unsaturated fatty acids. Science, n.s., **112:** 19–20, 1950.
5. Holmes, William: Silver staining of nerve axons in paraffin sections. Anat. Rec., **86:** 157–187, 1943.
6. Winkelmann, R. K.: Some sensory nerve endings in the skin. Arch. Dermat. & Syph., 1954. (In press.)